

## Identification of histamine receptor subtypes in skeletal myogenesis

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of H<sub>2</sub>R in the relaxation of acetylcholine-stimulated contraction of muscle cells, following the activation of professional histamine-producing cells, including mast cells. By contrast, H<sub>3</sub>R may participate in the regulation of specialized myocyte functions, potentially by maintaining the relaxed state under high and low histamine concentrations, locally produced/released by non-professional histamine-producing cells.

Histamine is a well-known biogenic and cationic amine, which is synthesized, stored and released by professional histamine-synthesizing cells. Mast cells, basophils and enterochromaffin cells (ECs) are the main histamine-producing cells in the body. Histamine is synthesized from L-histidine by histidine decarboxylase (HDC), which converts L-histidine to histamine (1). Histamine is released into and stored within storage granules, prior to regulated release (1). Following activation of professional histamine-producing cells, a burst release results in a transient high histamine concentration in the extracellular space. Histamine binds to and stimulates the conventional histamine receptors, histamine  $H_1$  and  $H_2$  receptors ( $K_d = 4.2$  nM) and histamine receptor type 2 ( $H_2S$ ,  $K_d = 4.3$  nM) (2). Smooth muscle cells (3,4), cardiomyocytes (5,6) and skeletal muscle tissue (7) express these conventional histamine receptors, which regulate cellular proliferation and the contraction state of the cells stimulated via the histamine/ $H_1R$  or  $H_2R$  axes (3-6).

**Key words:** myogenesis, differentiation, histamine receptor type 3, histamine

Table I. Primer sequences used in reverse transcription-quantitative polymerase chain reaction and the corresponding amplicon lengths.

Gene	Forward primer	Reverse primer	Length (bp)
Des	5'-GCCCTCAAGGGCACCAACGA-3'	5'-TTGCTCGGGGCTGGTTTCTCG-3'	297
Myog	5'-CCCAACCAGCGGCTGCCTAA-3'	5'-GTAGGGTCAGCCGCGAGCAA-3'	245
Myh2	5'-AGCTGCACCTTCTCGTTTGCCA-3'	5'-CGGTCAGGGTCGCTCCTGCT-3'	261
H <sub>1</sub> R	5'-CACTGGAGGCTGCCCTTGTGC-3'	5'-CACCAGCAGGTTGAGGCCAC-3'	167
H <sub>2</sub> R	5'-TCCTAAGCGACCCGGTACAGC-3'	5'-ATGGAGACTGAGGCACTGCTGG-3'	208
H <sub>3</sub> R	5'-TTCGAGCCTCCGCACCCAGAA-3'	5'-GGTCCAACGGCCGGTCAGC-3'	118
H <sub>4</sub> R	5'-TGCTCAGGTCCCTTGGCATT-3'	5'-ACGTGAGGGATGTACAGAGGAATGG-3'	189
PBGD	5'-AAAGTGCCGTGGGAACCAGC-3'	5'-CAGCCACAGCCAGGACGATG-3'	156

Des, desmin; Myog, myogenin; Myh2, myosin heavy chain IIa; H<sub>n</sub>R, histamine receptor type n (n=1-4); PBGD, porphobilinogen deaminase.

histamine from the non-professional histamine synthesizing cells along the histamine concentration gradient to the extracellular space (8). Histamine concentrations achieved in this manner DUHQRWVXIALHQWVRVLPXODWHFRQYHQWLRQDOKVWDDHQWVWIRIFLHQFHV/LWWOH&BOIRQW8.DQV Therefore, this mechanism was hypothesized to represent an ancestral vestigium of a function that had become obsolete during phylogenesis. However, studies conducted within the last decade that focus on G-protein coupled receptors have revealed novel members of the histamine receptor family (2). These novel histamine receptors, histamine receptor type 3 (H<sub>3</sub>R; S<sub>i</sub> = 8.0) and histamine receptor type 4 (H<sub>4</sub>R; S<sub>i</sub> = 8.2), have >10,000-fold JUHDWHUDIALWIRUKVWDPLQHFPSDUHGZUNPMHFXOWXUHSODWHDWFRQXHQFH conventional receptors (2). In addition, the low basal levels of histamine produced by non-professional histamine-producing cells, including dendritic cells (9) and lymphocytes (10,11), KYHEHHQGHPRQVWUDWHGWREHVXIALHQWLQRUGHUWRELOQSWROIC2 myogenesis, 50,000 cells/well were regulate cells equipped with these novel, high-DIALWKVWD seeded in 12-well plates (CellStar; Greiner Bio-One, Frickenhausen, Germany). The cells were initially grown in JURZWVHGLXPIRUVGZVWRUHDFFRQXHQFH1H[W the medium was exchanged with differentiation medium to induce myogenesis. Total RNA was isolated from the cells at days 0, 2, 4 and 6 using an RNeasy Mini kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. Total RNA (1 µg) was reverse transcribed using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RT-qPCR was performed with QJNVVWVWUDQGF1XVLQJL46<6Green Supermix (Bio-Rad Laboratories, Inc.) in an iCycler iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). Primers for mouse desmin (Des), myogenin (Myog), myosin heavy chain IIa (Myh2), H<sub>1</sub>R, H<sub>2</sub>R, H<sub>3</sub>R, H<sub>4</sub>R and porphobilinogen deaminase (PBGD) genes were designed using the National Center for Biotechnology Information Primer-Blast tool (Table I; <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>; accessed: 01/03/2012). The mRNA copy numbers of the samples analyzed were determined in triplicate and normalized against the PBGD gene.

#### ODWHULDOVDQGPHWKGV

**Cell culture.** The present study was approved by the institutional Medical Ethics Committee of the Institute of Clinical Medicine, University of Helsinki (Helsinki, Finland) and was performed in accordance with the 1983 Declaration of Helsinki. Mouse C2C12 myoblasts were obtained from the Turku Center for Biotechnology, University of Turku (Turku, Finland) (12), and maintained in growth medium

FRPSULVLQJ'XOEHFFRVPRGLNG(DJOHVPHGLXP'0(0 Lonza/BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, DQG P0 /JOXWDPLQJDDW&LQDKPLGLNG 5% CO<sub>2</sub> atmosphere. The composition of the differentiation medium was similar to the growth medium, with the exception of FBS, which was reduced from 10% to 1%. The cells were passaged using trypsinization (0.5% trypsin in 0.5 mM EDTA; Gibco-BRL Life Technologies, Carlsbad, CA, USA) ZUNPMHFXOWXUHSODWHDWFRQXHQFH

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** To investigate the expression of histamine WRELOQSWROIC2 myogenesis, 50,000 cells/well were seeded in 12-well plates (CellStar; Greiner Bio-One, Frickenhausen, Germany). The cells were initially grown in JURZWVHGLXPIRUVGZVWRUHDFFRQXHQFH1H[W the medium was exchanged with differentiation medium to induce myogenesis. Total RNA was isolated from the cells at days 0, 2, 4 and 6 using an RNeasy Mini kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. Total RNA (1 µg) was reverse transcribed using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RT-qPCR was performed with QJNVVWVWUDQGF1XVLQJL46<6Green Supermix (Bio-Rad Laboratories, Inc.) in an iCycler iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). Primers for mouse desmin (Des), myogenin (Myog), myosin heavy chain IIa (Myh2), H<sub>1</sub>R, H<sub>2</sub>R, H<sub>3</sub>R, H<sub>4</sub>R and porphobilinogen deaminase (PBGD) genes were designed using the National Center for Biotechnology Information Primer-Blast tool (Table I; <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>; accessed: 01/03/2012). The mRNA copy numbers of the samples analyzed were determined in triplicate and normalized against the PBGD gene.

,PPXQRXRUHVHFHQFHVWDDHQWVWIRIFLHQFHV12 cells were seeded at 2x10<sup>4</sup> cells/well in 24-well plates (CellStar) on coverslips and grown in growth medium for two days to reach 80% confluence, followed by culturing in differentiation

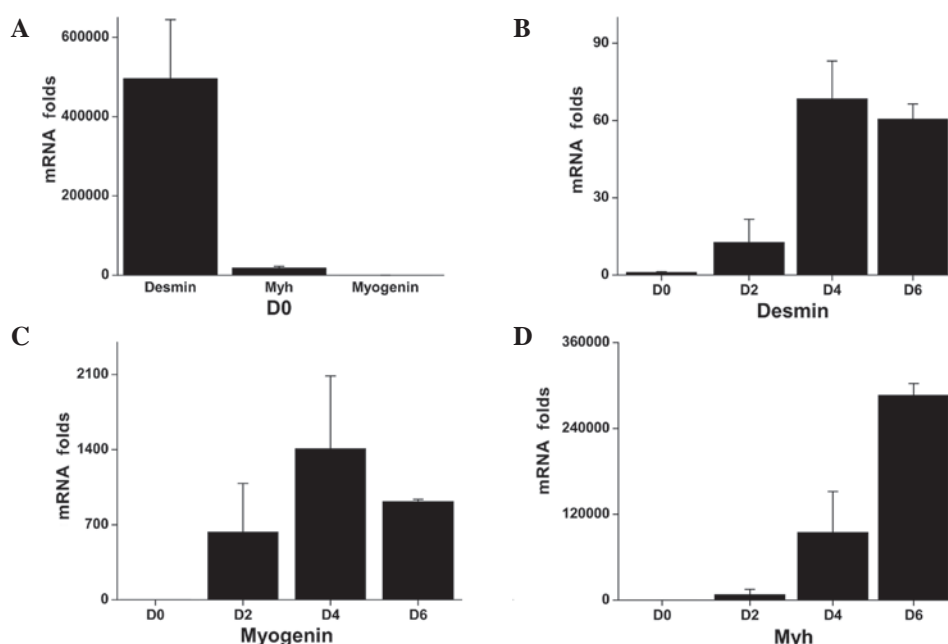


Figure 1. Myogenesis differentiation marker expression levels, determined by reverse transcription-quantitative polymerase chain reaction. (A) Relative expression levels of myogenic differentiation markers desmin (early marker), myogenin (intermediate marker) and Myh (late marker) in C2C12 cells at day 0, and relative expression level variations in (B) desmin, (C) myogenin and (D) Myh during myogenesis at days 2, 4 and 6. The data are expressed as the mean  $\pm$  standard deviation. Myh, myosin heavy chain; mRNA, messenger RNA; D, day.

medium to induce myogenesis. Differentiated cells from GDV DQG ZHUH HIGIRU PLQLQ SDUDIRU maldehyde (Sigma-Aldrich, St. Louis, MO, USA) with phosphate-buffered saline (PBS; 10mM phosphate buffer, 140 mM saline; pH 7.4), washed three times in PBS (5 min HDFWLPDQGLQULWRQ 7KUPRLVHU6FLHQWLÀ Fair Lawn, NJ, USA)/PBS for 15 min to permeabilize the cells. Subsequently, the cells were cultured under the following conditions sequentially: i) 10% normal donkey serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 1 h; ii) 1  $\mu$ g/POSROFORQDOSHSWLGHDIQLWSXULÀNORDHEHWLVRI & FHOONT-qPCR was used to detect the mRNA expression levels of the early, intermediate and late myogenesis markers, desmin, myogenin and Myh2, respectively, during differentiation. On day 0, desmin was expressed in myoblasts at significantly higher levels compared with the myogenin or Myh (Fig. 1A). The desmin expression levels increased during myogenesis, reaching 12-, 68- and 60-fold over the baseline level (day 0), on days 2, 4 and 6, respectively (Fig. 1B). On day 0, the myogenin mRNA expression levels were low; however, the mRNA expression levels increased by 631-, 1,408- and 914-fold at days 2, 4 and 6, respectively (Fig. 1C). Desmin and myogenin expression levels peaked on day 4, whereas the expression of Myh, a late myogenesis marker, continued to increase over the entire study period, reaching 7,718-, 94,487- and 286,288-fold higher expression levels at days 2, 4 and 6, respectively, compared with the baseline level (Fig. 1D).

Indirect immunofluorescence staining of the myogenesis marker proteins revealed positive staining of the early marker, desmin, at day 0 (Fig. 2A); however, no staining was observed for the intermediate marker, myogenin (Fig. 2B), or the late marker, Myh (data not shown). On day 2, staining for myogenin was found to be positive (Fig. 3A), whereas staining for Myh remained negative (Fig. 3B). On

Statistical analysis. SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses in addition to Matlab (MathWorks, Inc., Natick, MA, USA), which was used to perform the Mann-Whitney U test. All values are presented as the mean  $\pm$  standard error of the mean.  $P < 0.05$  was considered to indicate a statistically

5HVXOWV



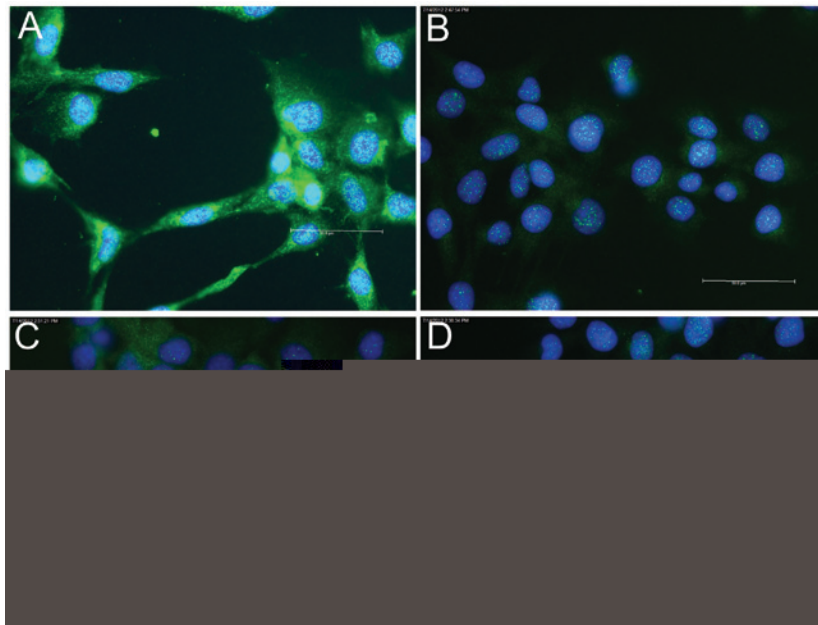


Figure 4. Staining of the late myogenic marker myogenin heavy chain was negative (not shown). DAPI was used for nuclear counterstaining (blue color). Scale bar, 50  $\mu$ m.

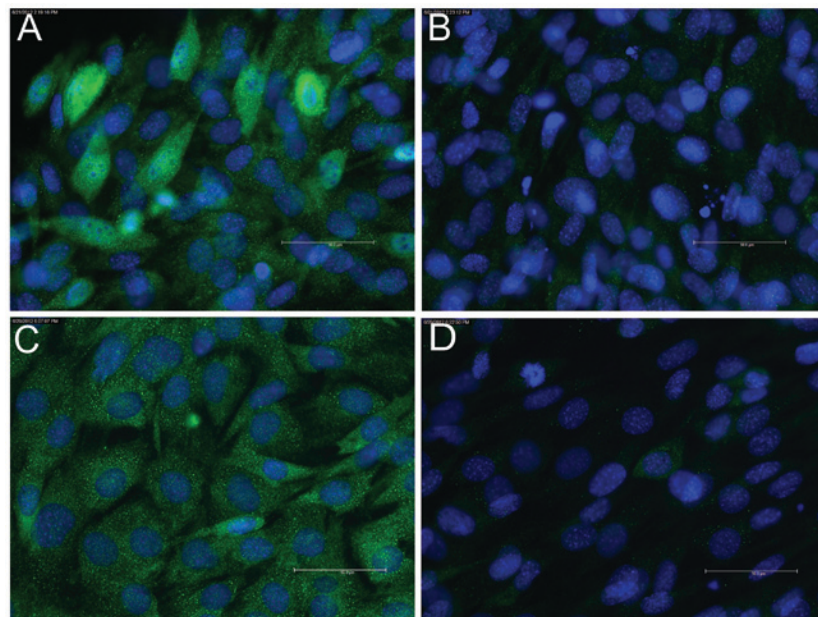


Figure 5. Differentiated C2C12 cells. DAPI was used for nuclear counterstaining (blue color). Scale bar, 50  $\mu$ m.

days 4 (data not shown) and 6, positive staining for myogenin (Fig. 4A) and Myh (Fig. 4B) was detected.

RT-PCR was used to detect the mRNA expression levels of histamine receptors associated with the differentiation stages (Fig. 5).  $H_1R$  mRNA was found to be highly expressed in C2C12 myoblasts (day 0), whereas expression was decreased during the differentiation process (Fig. 5A and B). By day 6, the expression level decreased to ~25% of the baseline level (day 0).  $H_2R$  mRNA was also expressed in C2C12 cells and the expression levels remained relatively

constant throughout the differentiation process (Fig. 5A and C). The expression of  $H_3R$  was found to be low in C2C12 myoblasts; however, following differentiation, the expression levels increased by 28-, 103- and 198-fold over the baseline level on days 2, 4 and 6, respectively (Fig. 5A and D).  $H_4R$  mRNA expression was not detected at any time-point.

Western blot analysis of C2C12 cells revealed almost negative staining at day 0 (Fig. 2C), weakly positive staining on day 2 (Fig. 3C) and strongly positive staining on days 4 (data not shown) and 6 (Fig. 4C).





